

EXTENDED REPORT

Trefoil factor family 1, MUC5AC and human leucocyte antigen-DR expression by conjunctival cells in patients with glaucoma treated with chronic drugs: could these markers predict the success of glaucoma surgery?

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Aims: To evaluate conjunctival expression of trefoil factor family (TFF)1, MUC5AC and human leucocyte antigen (HLA)-DR in patients with glaucoma treated with topical drugs, and to determine whether these parameters can predict the outcome of glaucoma surgery.

Methods: 77 conjunctival impression cytology specimens were collected from 77 patients with glaucoma (66 receiving drops with preservative and 11 treated with preservative-free drops) and 43 controls. TFF1, MUC5AC and HLA-DR expression was analysed using flow cytometry. Trabeculectomy was performed in 56 patients; success was defined as an intraocular pressure (IOP) ≤ 15 mm Hg without any IOP-lowering drug at 6 months.

Results: The expression of TFF1, MUC5AC and HLA-DR was significantly higher in patients than in controls ($p = 0.01$, 0.05 and 0.004 , respectively). A higher expression of MUC5AC was found in patients treated with preserved drops than in those receiving unpreserved drops ($p = 0.04$). A higher MUC5AC expression and a lower HLA-DR expression was observed in successful glaucoma surgeries than in failures.

Conclusions: TFF1 and MUC5AC secretions are probably a response to mild ocular surface changes caused by long-term use of topical treatment. Their increased expression could be a predicting factor of further successful glaucoma surgery.

Several studies have reported toxic side effects of anti-glaucoma drugs on the conjunctiva,^{1–3} especially if preservatives are used.⁴ Multiple and long-term topical treatment has been identified as a major risk factor for failure of filtering surgery.^{5–6}

Trefoil factor family (TFF) peptides are characterised by a three-loop structure and are typical secretory products of mucous epithelia. Although their mechanisms of action remain unclear, TFFs are coexpressed with and act in concert with gel-forming mucins.⁷ They link mucins and thereby influence the rheological properties of the mucus. TFF1 is secreted in the same blister of secretion as MUC5AC,⁸ a goblet cell-derived gel-forming mucin. TFFs are associated with protection and healing of mucosal tissue. These properties can be explained through the mitogenic and anti-apoptotic activities of TFF.⁹ Inflammatory reactions or ulcerations of the gastrointestinal tract increase TFF expression.⁹ During severe ocular inflammation such as pemphigoid or severe dry eye, TFF1 and MUC5AC expression decreases,¹⁰ as does goblet-cell density.¹¹ Moreover, there is growing evidence that conjunctival inflammation, assessed by human leucocyte antigen (HLA)-DR expression, is negatively correlated with MUC5AC production.¹²

To evaluate the drug-induced inflammatory reaction, we assessed the conjunctival expression of TFF1, MUC5AC and HLA-DR in patients who were treated for chronic glaucoma, and studied whether these parameters could predict the outcome of glaucoma surgery.

MATERIALS AND METHODS

The protocol of this prospective, observational, case-control study was approved by the local ethics committee of the Burgundy region, located in Dijon, France.

After fully informed consent was obtained, we took impression cytology specimens of 77 eyes of 77 patients with glaucoma. All were Caucasians and had primary open-angle glaucoma. They had been treated with at least one drop of drug (preserved or not) for more than 6 months.

Of these patients, 56 underwent a trabeculectomy. Impression cytology specimens were collected the day before surgery. Surgical success was defined as an intraocular pressure (IOP) ≤ 15 mm Hg without drugs, whereas failure was defined as an IOP > 15 mm Hg with or without drugs at 6 months.

At the same time, we took impression cytology specimens from 43 healthy people with no conjunctival disorders or topical treatment.

Conjunctival cells were collected using 20- μ m polyether sulfone filters (Supor Membranes, Gelman Sciences, Ann Arbor, Michigan, USA), as described previously.¹³ In each eye, two filters were applied successively, without exerting any pressure, on the supranasal and supertemporal bulbar conjunctiva, in two different but neighbouring areas. Membranes were immediately removed and dipped into a tube with 1.5 ml of cold phosphate-buffered saline (PBS; pH 7.4) with fixative (0.05% paraformaldehyde) prepared monthly. The specimens were kept at 4°C for 5 days, then gently agitated for 30 min and centrifuged (1600 rpm, 5 min) before processing for flow cytometry.¹³ Cells reacted for 30 min with fluorescein isothiocyanate-conjugated anti-HLA-DR (Dako SA, Copenhagen, Denmark), anti-TFF1 (P2802, provided by Dr MC Rio, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM,

Abbreviations: HLA, human leucocyte antigen; IOP, intraocular pressure; PBS, phosphate-buffered saline; TFF, trefoil factor family

Strasbourg, France), anti-MUC5AC (provided by Dr J Bara, INSERM, Saint Antoine Hospital, Paris, France) and iso-thiocyanate-conjugated anti-mouse immunoglobulin G1 (Immunotech, Marseille, France), as a negative control. Cell suspensions were then centrifuged in PBS (1600 rpm, 5 min), resuspended in 400 µl of PBS, and analysed on a flow cytometer (fluorescence-activated cell sorter (FACScan), Becton Dickinson, Mountain View, California, USA), equipped with an argon laser emitting at 488 nm. The linear plot showing cellular density (log side scatter) versus cell size (forward-angle light scatter) showed a single cell population. Analytic gates were set around this population to exclude cellular debris. The upper limit of fluorescence intensity obtained for the control antibody was considered to be the threshold of positivity for the three antibodies tested. At least 1000 cells were analysed for each marker and poorer specimens were discarded. Fluorescence levels of MUC5AC, TFF1 and HLA-DR were then calculated in each specimen. Results were expressed as the ratio of mean fluorescence intensity (MFI) (MFI marker/MFI isotypic control).

Owing to the sample size, non-parametric tests were used. Statistical values of the fluorescence ratio (mean (standard error mean (SEM))) were calculated in each group. A non-parametric Mann Whitney U test was carried out to compare the different groups. The Spearman's test was carried out to assess the correlation between TFF1 and MUC5AC. Values of $p < 0.05$ were considered to be significant.

RESULTS

Population

Among the 77 patients receiving anti-glaucoma drops, two groups were defined:

- Group A with 11 patients treated only with preservative-free drops (the active principle was a β -blocker).
- Group B with 66 patients treated with at least one preserved topical drug (the preservative was benzalkonium chloride). The number of patients treated with 1, 2, 3 or 4 associated drugs was 27, 31, 13 and 6, respectively. Prostaglandins were the most frequently prescribed ($n = 51$), followed by β -blockers ($n = 48$), α -2 agonists ($n = 19$), carbonic anhydrase inhibitors ($n = 28$) and miotics ($n = 6$).

Among the 56 patients operated on, and according to our criteria, 47 trabeculectomies were successful and 9 were considered failures (table 1).

The subjects included in the control group were significantly younger than those in the other groups (Kruskal-Wallis test, $p = 0.01$).

Conjunctival marker expression according to treatment

The expression of TFF1, MUC5AC and HLA-DR in glaucoma patients was significantly higher than that in controls (fig 1). A weak but significant correlation was found between TFF1 and MUC5AC expression in the treated group ($r = 0.31$; $p = 0.007$) and in the controls as well ($r = 0.31$; $p = 0.047$).

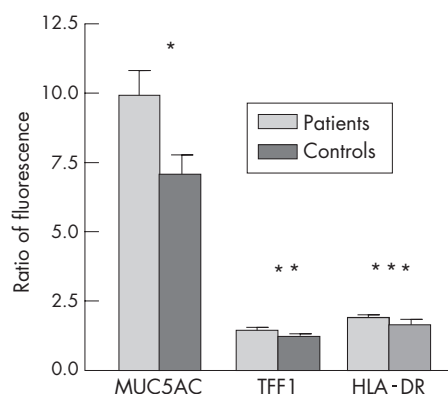


Figure 1 Conjunctival marker expression according to treatment (mean fluorescence intensity (standard error mean (SEM))). * $p = 0.05$; ** $p = 0.01$; *** $p < 0.01$. HLA, human leucocyte antigen; TFF, trefoil factor family.

Among these three markers, MUC5AC was the only one to be higher in patients receiving three different treatments (13.23 (8.51)) than in patients treated with monotherapy (6.99 (4.51); $p = 0.03$).

Conjunctival marker expression according to the presence of preservative

MUC5AC expression was higher in group B (preserved drops) than in group A (unpreserved drops; $p = 0.04$), whereas there was no significant difference for TFF1 and HLA-DR expression between the two groups ($p = 0.13$ and 0.94 , respectively; fig 2). TFF1 expression was correlated with MUC5AC in group B ($r = 0.41$; $p = 0.001$) but not in group A ($r = -0.16$; $p = 0.630$).

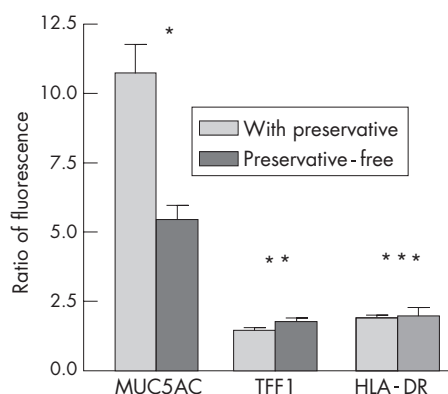


Figure 2 Conjunctival marker expression according to presence of preservative (mean fluorescence intensity (standard error mean (SEM))). * $p = 0.04$; ** $p = 0.13$; *** $p = 0.94$. HLA, human leucocyte antigen; TFF, trefoil factor family.

Table 1 Patient characteristics

	Controls, n = 43	Surgical group, n = 56	Topical treatment, n = 77	Preserved drops, n = 77	Unpreserved drops, n = 11
Sex ratio (male/female)	1.54	1.24	1.41	1.54	0.83
Age, mean (SD)	54.9 (23.2)	68.0 (13.0)	67.3 (12.9)	67.6 (13.5)	65.4 (9.3)

Conjunctival marker expression according to surgery efficacy

MUC5AC expression was higher in the success group than in the failure group ($p = 0.03$; fig 3). This was not observed for TFF1 values ($p = 0.11$). HLA-DR expression was lower in the group with effective surgery than in the group with failed surgery ($p = 0.04$). TFF1 expression was correlated with MUC5AC in the successful surgery group ($r = 0.39$; $p = 0.008$) but not in the failed surgery group ($r = 0.15$; $p = 0.710$).

DISCUSSION

Most patients with glaucoma are treated with one or several topical drugs for many years, and are therefore particularly exposed to the adverse effects of local drugs. Several *in vivo* and *in vitro* studies have shown that preservatives (the most common is benzalkonium chloride) are responsible for a large share of these toxic side effects. With the use of preserved eye drops, patients may experience ocular irritation, conjunctival redness, conjunctival follicles, fluorescein staining in the nasal bulbar conjunctiva, punctate keratitis, blepharitis,⁴ fibrosis,¹⁴ dry eye syndrome¹⁵ and reduced break-up time.¹⁶ Long-term use of topical preserved drugs induces histopathological conjunctival disorders, such as conjunctival metaplasia, a marked increase in pale cells, macrophages, mast cells, fibroblasts, a decrease in goblet cells in the substantia propria, and an increase in the thickness and number of epithelial cell layers.^{1 17 18}

Long-term and multiple topical drugs have been identified as a significant risk factor for trabeculectomy failure.^{3 6} A prolonged anti-glaucoma drug induces immune and inflammatory cell infiltration, which may be responsible for bleb fibrosis, the most common cause of filtration surgery failure. Considering the strong evidence for preservative toxicity, benzalkonium chloride is probably associated with the fibrosis process and bleb failure.¹⁹

In vitro, benzalkonium chloride induced oxidative stress, an overexpression of apoptotic marker, and decreased viability of Chang conjunctival cells.²⁰ Benzalkonium chloride showed a cellular toxicity at lower concentrations than those found in commercial preparations.²⁰ Moreover, a decrease in goblet cell density and mucus granule was observed in chronically treated patients.²¹

Our study showed an increase in the expression of HLA-DR by the conjunctival cells of treated patients, reflecting a subclinical inflammatory and immune reaction, which is in accordance with the literature.^{12 22 23} The mucous markers TFF1 and MUC5AC were overexpressed in these patients.

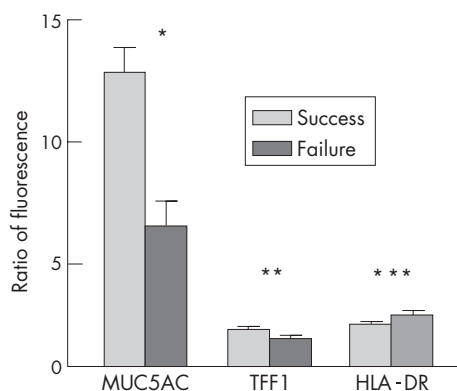


Figure 3 Conjunctival marker expression according to the outcome of filtering surgery (mean fluorescence intensity (standard mean error (SEM))). * $p = 0.03$; ** $p = 0.11$; *** $p = 0.04$. HLA, human leucocyte antigen; TFF, trefoil factor family.

Thus, trefoil peptides are coexpressed with mucins to protect mucosal epithelial cells and to trigger wound-healing responses in injured mucosa.⁷

In our study, we did not find a real difference in the expression of inflammatory markers between group A (preservative-free) and group B (with preservative); only MUC5AC varied significantly. However, unpreserved timolol may have toxicity in culture¹² and local instillations may have detectable effects on the ocular surface resulting from wash-off mucin,²⁴ which could explain the modification of the ocular surface even in the preservative-free group. Furthermore, TFF gene expression in cells is increased by hypotonicity and an increase in TFF1 secretion can depend on osmotic phenomena.²⁵ Moreover, of the 66 patients in group B, 51 were treated with a prostaglandin, which might have a protective effect.¹² However, our study is limited by the small size of the group without preservative ($n = 11$), which could explain the absence of TFF1 and HLA-DR expression and the difference with the preserved-drop group.

We observed a correlation between TFF1 and MUC5AC expression in all groups except in failed surgeries and the unpreserved-drop group. This may reflect the normal behaviour of goblet cells because they normally cosecrete these two factors in the same blisters of secretion.

In patients who underwent trabeculectomy, we found that lower HLA-DR expression was more frequent in successful surgeries than in failed surgical procedures. Moreover, surgical success was linked to a significant increase in MUC5AC and a trend towards increased TFF1 expression at baseline. This overexpression might result from an adapted response against the conjunctival aggression. In the failure group, these mechanisms might be ineffective. The overexpression of mucus markers might also result from a higher conjunctival goblet cell density, which has been related to good IOP control after filtering surgery; this has been proved with conjunctival biopsy²⁶ and more recently with confocal microscopy.²⁷

Further studies are needed to define the place of these markers as prognostic factors for filtering surgery. These markers could represent a new risk factor to take into account in the outcome of glaucoma surgery.

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Competing interests: None.

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Ethical approval: The protocol of this prospective, observational, case-control study was approved by the local ethics committee of the Burgundy region, located in Dijon, France.

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